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Sarraude, Tom; Hsu, Bin-Yan; Groothuis, Ton G. G.; Ruuskanen, Suvi

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BRIEF COMMUNICATION

Manipulation of Prenatal Thyroid Hormones Does Not Affect Growth or Physiology in Nestling Pied Flycatchers

Tom Sarraude[1,2,*]
Bin-Yan Hsu[1]
Ton G. G. Groothuis[2]
Suvi Ruuskanen[1]
[1]Department of Biology, FI-20014, University of Turku, Turku, Finland; [2]Groningen Institute for Evolutionary Life Sciences, University of Groningen, Nijenborgh 7, 9747 AG, Groningen, The Netherlands

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ABSTRACT

Hormones transferred from mothers to their offspring are thought to be a tool for mothers to prepare their progeny for expected environmental conditions, thus increasing fitness. Thyroid hormones (THs) are crucial across vertebrates for embryonic and postnatal development and metabolism. Yet yolk THs have mostly been ignored in the context of hormone-mediated maternal effects. In addition, the few studies on maternal THs have yielded contrasting results that could be attributed to either species or environmental differences. In this study, we experimentally elevated yolk THs (within the natural range) in a wild population of a migratory passerine, the European pied flycatcher (*Ficedula hypoleuca*), and assessed the effects on hatching success, nestling survival, growth, and oxidative status (lipid peroxidation, antioxidant enzyme activity, and oxidative balance). We also sought to compare our results with those of a closely related species, the collared flycatcher (*Ficedula albicolis*), that has strong ecological and life-history similarities with our species. We found no effects of yolk THs on any of the responses measured. We could detect only a weak trend on growth: elevated yolk THs tended to increase growth during the second week after hatching. Our results contradict the findings of previous studies, including those of the collared flycatcher. However, differences in fledging success and nestling growth between both species in the same year suggest a context-dependent influence of the treatment. This study should stimulate more research on maternal effects mediated by THs and their potential context-dependent effects.

*Corresponding author; email: t.sarraude@rug.nl.

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Introduction

Maternal effects are all the nongenetic influences of a mother on her offspring, and they are receiving increasing attention in evolutionary and behavioral ecology (Moore et al. 2019; Yin et al. 2019). Via maternal effects, mothers may influence the fitness of their progeny by adapting their phenotypes to expected environmental conditions (Mousseau and Fox 1998; "adaptive maternal effects" in Marshall and Uller 2007), and a recent meta-analysis found strong support for adaptive effects (Yin et al. 2019). Maternal effects are observed in plants, invertebrates, and vertebrates, and they can have many possible mediators (Danchin et al. 2011; Kuijper and Johnstone 2018). One intriguing pathway is via the hormones transmitted from the mother to her progeny. These hormone-mediated maternal effects have been found to profoundly influence offspring phenotypes in many different taxa (e.g., mammals [Dantzer et al. 2013], birds [von Engelhardt and Groothuis 2011], reptiles [Uller et al. 2007], and invertebrates [Schwander et al. 2008]). Most studies in the field of hormone-mediated maternal effects have focused on steroid hormones, such as glucocorticoids and androgens (Groothuis and Schwabl 2008; von Engelhardt and Groothuis 2011). However, mothers transfer other hormones to their embryos (Williams and Groothuis 2015), including thyroid hormones (THs; Ruuskanen and Hsu 2018).

THs are metabolic hormones produced by the thyroid gland and are present in two main forms: thyroxine (T_4) and triiodothyronine (T_3). T_3 has a greater affinity with TH receptors and is therefore responsible for most of the receptor-mediated effects. T_4, on the other hand, is mostly a precursor of T_3, although it may carry nongenomic effects (i.e., independent of TH receptors; Davis et al. 2016). THs have pleiotropic effects that serve several biologically important functions across vertebrates (Ruuskanen and Hsu 2018) and have been previously studied to some extent in various taxa (e.g., birds [Wilson and McNabb 1997], fish [Brown et al. 1988], and amphibians [Duarte-Guterman et al. 2010]). In early life, THs participate in the maturation of multiple tissues (e.g., birds [McNabb and Darras 2015] and mammals [Pascual and Aranda 2013]) and interact with growth hormones to increase growth (e.g., structural growth; Wilson and McNabb 1997; McNabb and

Darras 2015). THs also regulate metabolism and are necessary during adult life for normal reproductive functions (e.g., birds [McNabb and Darras 2015] and mammals [Norris and Carr 2013]). In wild bird species, plasma THs correlate positively with metabolic rate (Elliott et al. 2013; Welcker et al. 2013), and studies of mammalian model species found mechanistic evidence of the influence of THs on metabolism (Mullur et al. 2014). THs can alter the concentration of sodium and potassium in the cells (Haber and Loeb 1986; Ismail-Beigi et al. 1986), thereby requiring ATP consumption to restore a normal gradient, which in turn stimulates metabolism (Mullur et al. 2014).

THs could further influence cell oxidative status, a biomarker that may underlie life-history trade-offs and aging (Metcalfe and Alonso-Alvarez 2010) via multiple pathways. Oxidative stress occurs when the reactive oxygen species (ROS) production exceeds the capacity of antioxidant defenses (Monaghan et al. 2009). It results in oxidative damage on, for example, DNA, lipids, and proteins (Monaghan et al. 2009). Previous studies have shown that accelerated growth could increase oxidative stress (e.g., Alonso-Alvarez et al. 2007; Stier et al. 2014), and the stimulating effects of THs on growth and metabolism likely contribute to the production of ROS, thereby increasing oxidative stress (Asayama et al. 1987; Villanueva et al. 2013).

Studies of the effect of maternal THs on offspring development in wild animals are scarce. In humans and rats, a hypothyroid condition of the mother impairs brain development and cognition in her children (Moog et al. 2017). A potential problem here is that in mammalian species, maternal thyroid variation or manipulation inevitably influences other aspects of maternal physiology, which confounds the direct effects on the offspring. Oviparous species, such as birds, are therefore suitable models for studying the role of maternal hormones on progeny because embryos develop in eggs outside the mother's body and maternally derived hormones are deposited in egg yolks (Prati et al. 1992; Schwabl 1993). This allows the measurement and experimental manipulation of maternal hormone transfer to be independent of maternal physiology. Birds, with their relatively well-known ecology and evolution, have become the most extensively studied taxa in research on the function of maternal hormones (Groothuis et al. 2019).

Maternal THs have long been detected in the egg yolks of chickens (Hilfer and Searls 1980; Prati et al. 1992) and Japanese quails (Wilson and McNabb 1997). To date, only three studies have investigated the effects of physiological variation in yolk THs on offspring development (great tits, *Parus major* [Ruuskanen et al. 2016]; rock pigeons, *Columba livia* [Hsu et al. 2017]; collared flycatchers, *Ficedula albicollis* [Hsu et al. 2019]). These studies revealed potential biological relevance and fitness consequences but also some discrepancies in the role of yolk THs. For example, yolk THs improved hatching success in rock pigeons (Hsu et al. 2017) and in collared flycatchers (Hsu et al. 2019) but had no effect in great tits (Ruuskanen et al. 2016). Moreover, TH injection in great tit eggs increased offspring growth in males but decreased it in females (Ruuskanen et al. 2016). Conversely, yolk THs decreased growth during the second half of the nestling phase in rock pigeons (Hsu et al. 2017), whereas they increased early growth but decreased later postnatal growth in collared flycatchers (Hsu et al. 2019). Finally, resting metabolic rate (RMR) in great tits showed no response to elevated yolk THs (Ruuskanen et al. 2016), whereas RMR increased in females but decreased in male rock pigeon hatchlings (Hsu et al. 2017). These studies suggest that yolk THs may exert costs and benefits on the offspring in a species-specific manner. Another non–mutually exclusive hypothesis is that yolk THs may have context-dependent effects if the costs and benefits of THs differ across environments. For example, if prenatal THs increase RMR (as suggested by Hsu et al. [2017]), the elevated RMR may lead to increased growth in benign conditions but to decreased growth when resource availability is poor (Auer et al. 2015). Therefore, further studies on other species and contexts are needed to understand these contradicting findings.

Moreover, the study on collared flycatchers is the only one so far that investigated the association between yolk THs and oxidative stress in offspring (Hsu et al. 2019). This study surprisingly showed no adverse effect of yolk THs on whole-blood oxidative damage or oxidative balance, despite the early growth-enhancing effects found in the same study (Hsu et al. 2019). This absence of influence on oxidative stress contradicts the general knowledge of THs, with hyperthyroid tissues exhibiting higher oxidative damage in mammals (liver and heart [Venditti et al. 1997]; brain [Adamo et al. 1989]), calling for additional studies to confirm or contradict these findings.

To explore the origin of the discrepancies between previous studies (i.e., species or context dependency), we conducted an experiment similar to Hsu et al. (2019) in a closely related species with a similar ecological niche, the pied flycatcher (*Ficedula hypoleuca*). Pied and collared flycatchers are sister species that have very similar life histories, reproductive ecology, and morphology, and they can also hybridize (Lundberg and Alatalo 1992). Importantly, the similarity between the two species offers us an opportunity to explore the potential role of the environment in modulating the effect of maternal hormones, which may contribute to explaining the discrepancies of TH-related effects in the previous studies. To this end, we manipulated the concentrations of yolk THs in a wild population of pied flycatchers by injecting a combination of T_4 and T_3 into their eggs. We ensured that the treatment was within the physiological range. We also collected data on temperature, precipitation, and fledging success of pied flycatchers as proxies for environmental quality. These data were then compared with those collected previously for collared flycatchers (Hsu et al 2019). If the environmental contexts were similar between the two studies, we would expect to observe similar effects of elevated yolk THs, namely enhanced embryo development, hatching success, body mass, and structural growth. By contrast, if the environmental context and the effects of elevated yolk THs differed between the studies, it would lend some support for the potential of context-dependent modulation. Finally, elevated yolk THs may result in higher oxidative stress (a general trend in the literature; e.g., Villanueva et al. 2013) either directly via the stimulating effects of THs on metabolism or indirectly via increased growth, or no association with oxidative stress may be shown at all (as suggested by Hsu et al. [2019]).

Material and Methods

Study Site and Study Species

The experiment was conducted during spring 2017 in Turku, southwest Finland (60°26′N, 22°10′E). The study species is the pied flycatcher, a small (ca. 15 g) migratory passerine that breeds in Finland from May to July. Pied flycatchers are secondary cavity nesters that also breed in artificial nest boxes. At this latitude, females generally lay a single clutch of five to eight eggs.

Nest Monitoring and Experimental Design

Yolk TH concentrations were elevated via injections in unincubated eggs using a between-clutch design (i.e., all eggs of the same clutch received the same injection). In total, 29 clutches (170 eggs) received a TH injection (hereafter, "TH treatment"), and 28 clutches (169 eggs) received a control injection (hereafter, "CO treatment"). In two nests, one in each treatment, none of the eggs hatched because of desertion before incubation. These two clutches were therefore removed from the analysis. The final sample size is 28 TH treatment nests (166 eggs) and 27 CO treatment nests (164 eggs).

Nest boxes were monitored twice a week during nest construction until egg laying. On the morning when the fifth or sixth egg was laid, all eggs were temporarily removed from the nest for injection, replaced with dummy eggs, and returned after injection. Nests were then visited every following morning to inject freshly laid eggs until clutch completion, marked by the absence of freshly laid eggs and females incubating their eggs. Females generally start incubating their eggs after the last egg has been laid.

The clutches were randomly assigned to one of the treatments. In addition, the treatments were alternated across clutches to balance the order of the treatments within a day. Similarly, we also balanced the treatments across the laying period. There was no difference in the average (±SD) laying date from May 1 (TH = 27.00 ± 2.64 vs. CO = 27.19 ± 2.65; Wilcoxon unpaired test: $W = 402.5$, $P = 0.68$) or in the average (±SD) clutch size (TH = 5.93 ± 0.81 vs. CO = 6.07 ± 0.78 eggs; Wilcoxon unpaired test: $W = 439.5$, $P = 0.26$).

Preparation of the Solution and Injection Procedure

The TH solution was composed of a mix of T_4 (L-thyroxine, ≥98% HPLC, CAS no. 51-48-9, Sigma-Aldrich) and T_3 (3,3′,5-triiodo-L-thyronine, >95% HPLC, CAS no. 6893-02-3, Sigma-Aldrich), first dissolved in 0.1 M NaOH and then diluted in 0.9% NaCl. The concentration of each hormone was based on hormone measurements in 15 pied flycatcher eggs collected from 15 clutches in spring 2016 in Turku. The average (±SD) hormone content of these eggs was as follows: T_4 = 2.307 ± 0.654 and T_3 = 0.740 ± 0.238 ng/yolk. We injected twice the standard deviation of each hormone (1.308 ng/yolk of T_4 and 0.477 ng/yolk of T_3), a standard and recommended procedure for hormone manipulation within the natural range (Ruuskanen et al. 2016; Hsu et al. 2017; Podmokła et al. 2018). The CO solution was a saline solution (0.9% NaCl).

Before the injection, the shell was disinfected with a cotton pad dipped in 70% alcohol. The injection procedure consisted of four steps. First, a disposable and sterile 25G needle (BD Microlance) was used to pierce the shell. To locate the yolk, the egg was lit by a small torch from underneath. Second, the injection of 5 μL was performed with a Hamilton syringe (25 μL) directly into the yolk. Third, the hole in the shell was sealed with a veterinary tissue adhesive (3M Vetbond), and the eggs were marked with a permanent marker (Stabilo OHPen universal). Finally, all eggs from a clutch were returned to the nest at the same time, and the dummy eggs were removed.

Nestling Growth Monitoring and Blood Sampling

Nests were checked daily for hatching 2 d before the expected hatching date. The date of hatching for a particular nest was recorded as the day the first hatchlings were observed (day 0). Two days after hatching, nestlings were coded by clipping down feathers to identify them individually. Nestlings were ringed at day 7 after hatching. Body mass (0.01 g) was recorded at days 2, 7, and 12 after hatching. Tarsus (0.1 mm) and wing length (1 mm) were recorded at days 7 and 12. At day 12, blood samples from all nestlings were also collected (ca. 40 μL) from the brachial vein in heparinized capillaries and directly frozen in liquid nitrogen for analyses of oxidative stress biomarkers and molecular sexing. All nestlings from the same nest were sampled within 20 min. Samples were stored at −80°C until analyses. Finally, fledging was monitored from day 14 after hatching. Fledging date was recorded when all the nestlings had fledged from the nest, and fledging success (fledged/not) was scored for each hatchling.

Finally, we collected data on temperature (hourly averages) and precipitation from the European Climate Assessment and Dataset (Klein Tank et al. 2002) and calculated the daily averages and length of periods of continuous rain, a key factor affecting mortality in flycatchers (Siikamäki 1996; Eeva et al. 2002). Temperature data (hourly averages) were extracted from a station located approximately 3 km from our field site. To compare environmental conditions between the collared flycatcher study by Hsu et al. (2019) and our study, we also collected similar data for the study period from a field station close to the collared flycatcher population (see figs. A1, A2; table A1). In addition, we used overall fledging success as a proxy for environmental quality. In both populations, nest predation and adult mortality rates are low and are not main determinants of fledging success (Doligez and Clobert 2003; B. Doligez and S. Ruuskanen, personal communication). Thus, fledging success may be a good indicator of environmental conditions during the nestling phase. The data in Hsu et al. (2019) and in our experiment were collected during the same year (2017), and both nest box populations were located in mixed-forest habitats.

Sexing Method

The DNA extraction procedure from the blood cells followed Aljanabi and Martinez (1997), using approximately 5 μL of

whole-blood samples. The method of sexing followed that described by Ruuskanen and Laaksonen (2010), with minor changes to the polymerase chain reaction (PCR) condition: 5-μL QIAGEN multiplex PCR kit, 0.1 μL of each primer (20 μM), 1.8 μL of H_2O, and 3 μL of DNA, yielding 10 μL for the final PCR volume. The initial denaturation was at 95°C for 15 min, followed by 35 cycles of 95°C for 30 s, 55°C for 90 s, and 72°C for 60 s. The samples were then held at 72°C for 10 min and 20°C for 5 min. PCR products were analyzed with 3% agarose gel under 100 V for 90 min.

Oxidative Stress Analysis Methods

Samples from two individuals per clutch were analyzed. Whenever possible, one male and one female with approximately the same body mass were chosen, since body mass is known to covary with oxidative status (Rainio et al. 2015). The average ($\pm$SD) difference in mean body mass between the chicks selected for oxidative stress analysis within each clutch is -0.01 ± 0.43 g (range $= -1.80$ to 0.77 g). If samples could not be taken for both sexes from a clutch, then two individuals of the same sex were selected. In total, 103 nestlings were included in the analysis (for TH, $N = 27$ nests and 50 nestlings; for CO, $N = 27$ nests and 53 nestlings).

Three biomarkers of oxidative status were measured: the activity of the antioxidant enzyme glutathione S-transferases (GSTs), the reduced-glutathione-to-oxidized-glutathione (GSH:GSSG) ratio, and lipid peroxidation (using malonaldehyde [MDA] as a proxy; Sheenan et al. 2001; Halliwell and Gutteridge 2015). GST enzymes catalyze the conjugation of toxic metabolites to glutathione (Sheenan et al. 2001; Halliwell and Gutteridge 2015). GST activity is expected to be lower in normal cells than in damaged cells (Rainio et al. 2013). The GSH:GSSG ratio represents the overall oxidative state of cells, and a low ratio reveals oxidative stress (e.g., Rainio et al. 2013, 2015; Halliwell and Gutteridge 2015). Lipid peroxidation is commonly measured with the thiobarbituric acid reactive substances (TBARS) test (Alonso-Alvarez et al. 2008; Halliwell and Gutteridge 2015). This test relies on the ability of polyunsaturated fatty acids contained in cell membranes to readily react with oxygen radicals by donating a hydrogen atom. The fatty acid radical is unstable, and a chain of reactions occurs. MDA is an end product of this reaction (Marnett 1999) and is thus used as a measure of lipid peroxidation.

Whole blood was first thawed and then diluted in 0.9% NaCl to achieve protein concentrations ranging 4–13 mg/mL. Overall protein concentration (mg/mL) was measured using a bicinchoninic acid protein assay (Thermo Scientific) with a bovine serum albumin standard (Sigma-Aldrich). The methodology for measuring GST and the GSH:GSSG ratio followed Rainio et al. (2015). The marker of lipid peroxidation, MDA, was analyzed using a 384-plate modification of the TBARS assay described by Espín et al. (2017). All biomarker enzyme activities were measured in triplicate (intraassay coefficient of variability is <10% in all cases).

Statistical Analysis

Data were analyzed with the software R version 3.5.3 (R Development Core Team 2019). General and generalized linear mixed models (LMMs and GLMMs, respectively) were performed using the R package lme4 (Bates et al. 2015). All mixed models included nest as a random intercept. *P* values in LMMs were obtained by model comparison using the Kenward-Roger approximation from the package pbkrtest (Halekoh and Højsgaard 2014). The significance of the predictors in GLMMs was determined by parametric bootstrapping with 1,000 simulations using the package pbkrtest. Model residuals were checked for normality and homogeneity by visual inspection. Significant interactions were further analyzed by post hoc comparison with the package phia (de Rosario-Martinez 2015). Estimated marginal means (EMMs) and standard errors were derived from models using the package emmeans (Lenth 2019).

To analyze hatching success, a dummy code was given to each egg: 0 for unhatched egg and 1 for hatched egg. A GLMM was performed with a binomial error distribution (logit link). Treatment was included as the predictor, and two covariates were included: the average temperature over the egg-laying period and clutch size. Fledging success was coded similarly: 0 for dead and 1 for fledged nestling. A similar GLMM was fitted, with treatment as a predictor and the average temperature over the nestling period and brood size at day 2 as covariates.

Duration of the embryonic period and duration of the nestling phase were fitted in separate linear models with treatment as the fixed effect and the average ambient temperature over these two phases as covariates to control for potential temperature-related effects (Olson et al. 2006; Salaberria et al. 2014). Laying date and brood size were added as additional covariates for nestling phase duration, as they both may influence nestling growth and thereby nestling phase duration (Williams 2012).

Early body mass (i.e., at day 2 after hatching) was analyzed separately from growth during the second week after hatching (i.e., days 7–12) for two reasons. First, variation in the former may better represent the influence of maternal THs on prenatal development, while variation in the latter also reflects the influence during the postnatal stage when the yolk that contains the hormones is totally consumed. Second, including the three time points in a single model created a nonlinear growth curve, hampering proper statistical analyses. The model to analyze early body mass included laying date and mean temperature between hatching and day 2 as covariates. To analyze growth between days 7 and 12, we used the scaled mass index (SMI) by Peig and Green (2009), a recommended method to estimate changes in body condition. The SMI was calculated as follows:

$$\mathrm{SMI}_i = M_i \times (L_0/L_i)^b,$$

where M_i and L_i are body mass and tarsus length of the individual i, respectively, L_0 is the mean value of tarsus length for the whole population ($L_0 = 17.0$ mm; $N = 228$), and b is the slope estimate of a regression of ln-transformed body mass on ln-transformed tarsus length ($b = 1.83$). Furthermore, we analyzed growth in wing and tarsus length separately, given that THs may also influence structural size (e.g., Wilson and McNabb 1997) independently of mass. Models used to analyze

the morphological variables included sex as a fixed factor to test for potential sex-dependent effects of THs, as found by Hsu et al. (2017) and Ruuskanen et al. (2016). Treatment and age were added as fixed factors together with their two- and three-way interactions with sex. Brood size at day 2, laying date, and average temperature were included as covariates. Individual identity was added as a random intercept to account for repeated measures.

The models used to analyze growth (SMI and structural size) included age and treatment as fixed factors. Brood size at day 2, laying date, and average daily temperature (between days 3 and 7 for measurements at day 7 and between days 8 and 12 for measurements at day 12) were added as covariates, and nestling identity was added as an additional random intercept to account for repeated measures.

The models of oxidative stress biomarkers (i.e., GST activity, MDA concentration, and GSH:GSSG ratio) included treatment and sex as the predictors and brood size at day 2, laying date, and mean daily temperature as covariates. Body mass at day 12, which was the day of blood sampling, was included as an additional covariate because body mass is known to be associated with oxidative status (e.g., Rainio et al. 2015). In a separate model, body mass at day 12 was replaced with growth rate (g/d) between days 7 and 12 to test the association of growth rate with oxidative stress. Assay number was also added as a random intercept to account for interassay variation. Response variables were log transformed to achieve normal distribution of the residuals.

Ethical Note

The study complied with Finnish regulation and was approved by the Finnish Animal Experiment Board (ESAVI/2389/04.10.07/2017) and by the Finnish Ministry of Environment (VARELY580/2017).

Results

Hatching and Fledging Success, Duration of Embryonic and Nestling Periods

Hatching success (TH = 75.3% vs. CO = 76.8%) and fledging success (TH = 92.2% vs. CO = 92.3%) were similar between the two treatments (GLMMs; $P > 0.71$). Hatching success was not affected by clutch size or by ambient temperature during incubation (GLMMs; both $P > 0.09$). Likewise, fledging success was not correlated with brood size at day 2 or with ambient temperature (GLMMs; both $P > 0.10$). Duration of the embryonic period did not differ between the groups ($t = -0.59$, $P = 0.56$). Injection of yolk THs did not affect the duration of the nestling period either ($t = -1.01$, $P = 0.32$). Likewise, there was no association between laying date or brood size at day 2 and the duration of the nestling period (all $t < 1.87$, $P > 0.07$). Finally, there was no association between temperature and duration of embryonic or nestling periods (all $P > 0.09$).

Growth

Experimental elevation of yolk THs did not affect early postnatal body mass (day 2 EMMs ± SE: CO = 3.63 ± 0.13 vs. TH = 3.50 ± 0.13 g) and neither did sex (table 1). We detected a tendency of an interaction between treatment and age on nestling SMI between days 7 and 12 that did not reach statistical significance ($P = 0.07$; table 1). Although the interaction was not significant, we performed post hoc analyses to explore the trend further. We found that TH-treated nestlings tended to grow faster than control nestlings during the second week after hatching (adjusted slope ± SE: for CO, 1.32 ± 0.11; for TH, 1.61 ± 0.12; $\chi^2 = 3.41$; Holm-adjusted $P = 0.06$; fig. 1). However, there was no significant difference in SMI between the treatments at day 7 ($\chi^2 = 0.06$, Holm-adjusted $P = 0.81$) or at day 12 ($\chi^2 = 1.04$, Holm-adjusted $P = 0.62$), indicating that the interaction likely originates from small differences in the opposite directions at days 7 and 12 between TH and control groups. On average, males had a slightly higher SMI than females between days 7 and 12 (EMMs ± SE: for males, 80.78 ± 0.59; for females, 79.86 ± 0.61; table 1). For structural size measurements, tarsus and wing lengths, however, no effects of yolk TH treatment were detected (table 1). Ambient temperature was negatively correlated with SMI and positively associated with wing length (table 1).

Oxidative Stress and Oxidative Damage

Experimental elevation of yolk THs did not affect antioxidant enzyme activity (mean ± SE: for CO and TH, 0.006 ± 0.0003 pmol GST/min/mg protein), oxidative damage on lipids (for CO, 0.051 ± 0.003 nmol MDA/mg protein; for TH, 0.053 ± 0.004 nmol MDA/mg protein), or oxidative status (GSH:GSSG ratio: for CO, 3.86 ± 0.47; for TH, 4.51 ± 0.73; table 2). None of the other predictors or covariates (i.e., sex, body mass, growth rate, temperature, and brood size) were associated with these oxidative stress biomarkers, except laying date, which was negatively correlated with MDA concentration (table 2).

Environmental Context

Patterns of temperature and precipitation during the different stages of breeding are shown in figures A1 and A2 for pied flycatchers and collared flycatchers. There were only minor differences in mean temperature across the stages and species: for pied flycatchers, the average temperatures over the laying, incubation, and nestling periods were 12.14°, 13.43°, and 15.02°C, respectively, and for collared flycatchers, they were 12.48°, 13.67°, and 14.56°C. Likewise, the number of days with rain was rather similar (table A1), and we could not reliably associate peaks in precipitation with peaks in nestling mortality (fig. A3). Importantly, however, collared flycatchers experienced lower fledging success (ca. 75%; Hsu et al. 2019) compared with pied flycatchers (ca. 90%) during the study year, whereas both species have similar fledging success of about 90% when the environmental conditions are good (Qvarnström et al. 2009), suggesting that the collared flycatchers

Table 1: Full linear mixed models of morphometric measures in response to yolk thyroid hormone elevation (TH treatment) in nestling pied flycatchers

	Body mass at day 2 (g)			Scale mass index days 7–12			Wing length days 7–12 (mm)			Tarsus length days 7–12 (mm)		
Predictor	E (SE)	F_{ddf}	P	E (SE)	F_{ddf}	P	E (SE)	F_{ddf}	P	E (SE)	F_{ddf}	P
Treat (TH)	−.15 (.17)	$.86_{49.0}$	.36	−3.06 (2.61)	$.007_{51.6}$	.94	.08 (.92)	$.10_{50.0}$	.75	.07 (.22)	$.44_{49.6}$	.51
Sex (male)	.04 (.05)	$.58_{182.5}$	.45	.58 (2.21)	$4.6_{188.2}$	.03	.51 (.69)	$1.15_{183.2}$	.29	.02 (.18)	$.03_{190.3}$	.86
Age (12 d)	…	…	…	1.30 (.17)	$330.4_{226.0}$	<.001	4.72 (.05)	$39{,}241_{226.0}$	<.001	.29 (.01)	$2{,}174.0_{226.0}$	<.001
Laying date	.004 (.04)	$.01_{52.9}$	.92	−.07 (.22)	$.1_{56.8}$	.77	.05 (.11)	$.17_{55.2}$	.69	.001 (.02)	$.004_{57.2}$	.95
Brood size	.16 (.07)	$5.46_{56.6}$	.02	−.90 (.47)	$4.3_{61.3}$	.04	.36 (.24)	$2.27_{58.3}$	.14	.08 (.05)	$3.24_{62.4}$	.08
Temperature	−.11 (.06)	$3.18_{49.9}$	.08	−.97 (.17)	$33.1_{236.9}$	<.001	.22 (.05)	$19.2_{227.8}$	<.001	−.004 (.01)	$.10_{231.7}$	.75
Treat × sex	…	…	…	1.15 (3.20)	$.1_{187.0}$	.76	−.66 (.99)	$.08_{182.1}$	.78	−.14 (.26)	$.07_{189.2}$	.80
Treat × age	…	…	…	.36 (.24)	$3.2_{224.2}$	.07	−.03 (.07)	$.06_{224.1}$	.81	−.01 (.02)	$.30_{224.1}$	.59
Sex × age	…	…	…	.03 (.22)	$.03_{224.0}$	.86	−.04 (.07)	$.001_{224.0}$	.98	−.0004 (.02)	$.18_{224.0}$	.67
Treat × age × sex	…	…	…	−.10 (.32)	$.1_{223.0}$	.72	.08 (.10)	$.70_{223.0}$	.41	.01 (.02)	$.23_{223.0}$	.63

Note. P values and denominator degrees of freedom (ddf) were obtained using the Kenward-Roger approximation (numerator degrees of freedom equal 1). P values were obtained by removing each predictor one by one from the model except for the main effects of treatment, sex, and age, which were removed from models without their interactions; otherwise, the models were not nested. $N = 125$ for TH treatment and 126 for control treatment. E = estimate; treat = treatment.

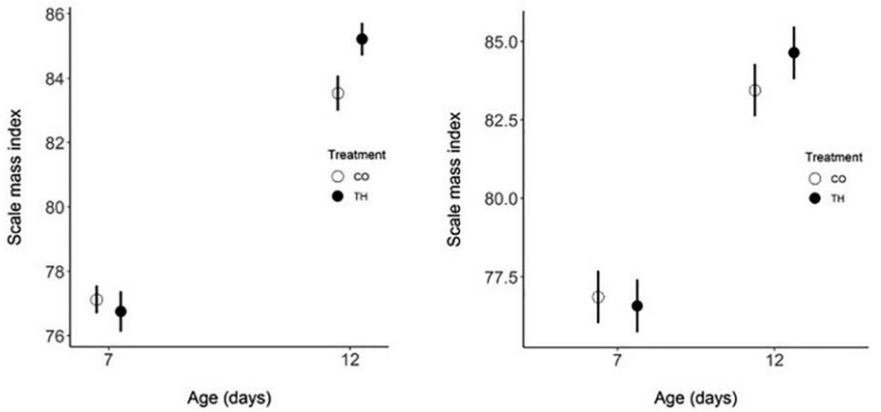

Figure 1. Scale mass index raw data (mean ± SE; *left*) and marginal means (*right*) at days 7 and 12 after hatching for nestlings in the yolk thyroid hormone elevation treatment (TH; $N = 125$) and the control treatment (CO; $N = 126$). The interaction between treatment and age of nestlings approached significance ($P = 0.07$).

experienced harsher environmental conditions than the pied flycatchers.

Discussion

We replicated an experimental study on the effect of egg THs on offspring development in collared flycatchers in a closely related and ecologically similar species, the pied flycatcher, while at the same time monitoring environmental factors. This would allow us to study the generality of results found earlier but also potential environmentally dependent hormone effects.

Overall, our results for pied flycatchers differ substantially from those for collared flycatchers (Hsu et al. 2019). We found no effect of prenatal THs on hatching success or growth (in body mass, body condition, or structural growth), whereas Hsu et al. (2019) found an increase in hatching success and in early growth but decreased growth during the second week of the nestling period. Because these two species are closely related and display ecological similarities (Lundberg and Alatalo 1992), we predicted that such discrepancies in the results could arise if THs influence growth differently in different environmental conditions. We observed that fledging success, a proxy for environmental harshness, was lower in the collared flycatcher experiment than in the pied flycatcher experiment. Yet temperatures and rainfall did not generally seem to differ across the studies, suggesting that other environmental factors may interact with yolk THs. Furthermore, collared flycatchers generally have a slightly higher early body mass (Qvarnström et al. 2009) and a higher fledging mass (Myhrvold et al. 2015) than pied flycatchers. Yet when comparing the present study with Hsu et al. (2019), collared flycatchers had a lower early body mass than pied flycatchers and a similar body mass close to fledging, suggesting poorer growth of collared flycatchers during the study year. Prenatal environmental conditions (i.e.,

Table 2: Full linear mixed models of oxidative stress biomarkers in response to yolk thyroid hormone elevation (TH treatment) in nestling pied flycatchers at day 12 after hatching

	MDA concentration (nmol/mg protein)			GSH:GSSG ratio			GST activity (pmol/min/mg protein)		
Predictor	E (SE)	F_{ddf}	P	E (SE)	F_{ddf}	P	E (SE)	F_{ddf}	P
Treat (TH)	.004 (.074)	$.003_{41.3}$	.96	.13 (.16)	$.63_{44.6}$	.43	−.07 (.06)	$1.21_{44.6}$	.28
Sex (male)	−.03 (.07)	$.18_{55.1}$	.67	.11 (.14)	$.55_{55.4}$	.46	−.07 (.06)	$1.73_{57.4}$	.19
Mass at day 12	−.06 (.04)	$2.78_{45.2}$	.10	−.07 (.08)	$.62_{60.6}$	.43	.02 (.03)	$.46_{49.4}$	.50
Temperature	−.006 (.019)	$.09_{42.3}$	.76	−.003 (.039)	$.008_{44.4}$	.93	.001 (.016)	$.003_{43.8}$	.95
Laying date	−.04 (.02)	$5.40_{40.2}$	.03	.02 (.03)	$.51_{49.8}$	.48	.005 (.014)	$.13_{42.9}$	.72
Brood size	−.06 (.03)	$3.13_{49.9}$	.08	.001 (.072)	$.0002_{59.1}$	.99	.001 (.028)	$.002_{52.7}$	.97
Growth rate[a]	−.15 (.15)	$.94_{51.2}$	.34	.23 (.35)	$.42_{66.3}$	.52	−.06 (.13)	$.17_{58.0}$	.68

Note. Response variables were log transformed to achieve normal distribution of the residuals. *P* values and denominator degrees of freedom (ddf) were obtained using the Kenward-Roger approximation (numerator degrees of freedom equal 1). To examine the association between growth rate (between days 7 and 12) and oxidative status, we further replaced body mass with growth rate in the reduced model while keeping all other predictors constant. *P* values of the predictors were obtained by removing these predictors individually from the full model. $N = 125$ for TH treatment and 126 for control treatment. E = estimate; treat = treatment; MDA = malonaldehyde; GSH = reduced glutathione; GSSG = oxidized glutathione; GST = glutathione S-transferase.

[a]Tested in a model other than body mass at day 12.

during egg laying and incubation) were rather similar between the two species and thus cannot explain why yolk THs enhanced hatching success and early body mass in collared flycatchers (Hsu et al. 2019) but not in pied flycatchers (this study). More experimental studies on the context-dependent effects of yolk THs are thus needed.

Despite no clear differences in temperature and precipitation, the lower growth and survival of nestling collared flycatchers suggest that the environmental conditions may have been harsher in this population than in the pied flycatcher population. Such environmental conditions may have contributed to the contrasting results on the effects of yolk THs on postnatal growth. We can speculate that a potential underlying mechanism is linked to metabolic rates. Hsu and colleagues suggested that prenatal THs increase basal metabolic rates (Hsu et al. 2017). Increased basal metabolic rates may lead to decreased postnatal growth in harsh conditions, such as those for the collared flycatcher population, but have no effect or even increase growth when resource availability is good, as is the case for the pied flycatcher population. Nevertheless, despite the high degree of ecological similarity between the two species, the possibility that species differences actually explained the contrasting results remains to be examined.

We observed no effect on antioxidant enzyme activity (GST) or in the oxidative balance (GSH:GSSG ratio) and no increase in oxidative damage in lipids (MDA) in response to elevated yolk THs. The earlier study on collared flycatchers reported similar levels of oxidative stress biomarkers and found no increase in oxidative stress in response to elevated prenatal THs (Hsu et al. 2019). These results may suggest that egg THs do not affect the oxidative status of nestlings as would be expected from the literature. However, the absence of detrimental consequences on oxidative stress may be due to the experimental design of both studies, with an increase in yolk THs within the natural range of the species. Thus, individuals may have been able to raise their antioxidant capacities (other than those measured in this study) to avoid oxidative damage. That said, physiological elevation (i.e., within the natural range) of yolk THs was necessary to get ecologically relevant results. Furthermore, because of fieldwork constraints, there are some limits to our approach. We measured a limited number of markers of oxidative status at a single time point in one tissue and therefore lack an overview of the variation that may have happened over the course of the whole nestling phase, as well as in other tissues and for other biomarkers. Further studies with more comprehensive measures of oxidative stress would help in understanding the relationship between yolk THs and oxidative stress.

In conclusion, this study shows no convincing effect of yolk THs on nestling development. We found that yolk THs did not increase growth, incurred no extra oxidative damage, and did not affect nestling survival. Our results differ from a study on a closely related species, suggesting that the role of prenatal THs may differ according to the environment experienced by the progeny. The study adds to the small body of literature on TH-mediated maternal effects, which have been largely neglected so far. Research on maternal THs would greatly benefit from further studies with the same species in different experimentally manipulated contexts. It would also profit from comparative studies on species with different life histories that are likely to influence the effects induced by exposure to maternal THs.

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APPENDIX

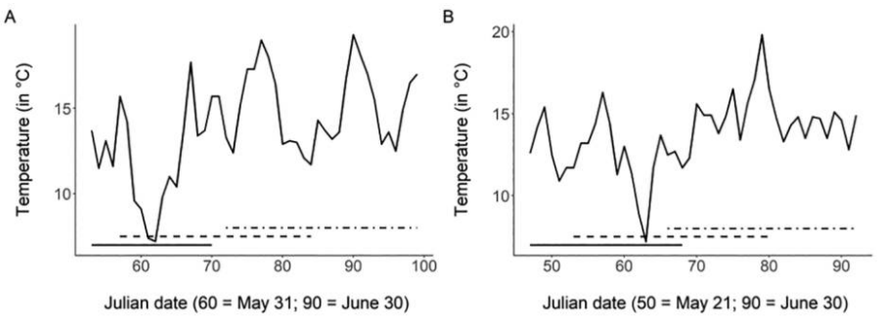

Figure A1. Average daily temperature experienced by pied flycatchers in Turku, Finland (*left*), and by collared flycatchers in Gotland, Sweden (*right*). Solid, dashed, and dash-dotted lines represent egg-laying, incubating, and nestling periods, respectively. In Turku, average temperatures over the different periods were 12.14°, 13.43°, and 15.02°C. In Gotland, average temperatures were 12.48°, 13.67°, and 14.56°C.

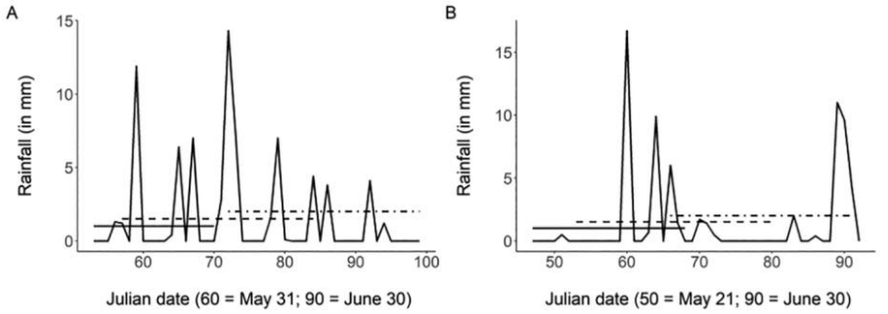

Figure A2. Daily precipitation experienced by pied flycatchers in Turku, Finland (*left*), and by collared flycatchers in Gotland, Sweden (*right*). Solid, dashed, and dash-dotted lines represent egg-laying, incubating, and nestling periods, respectively.

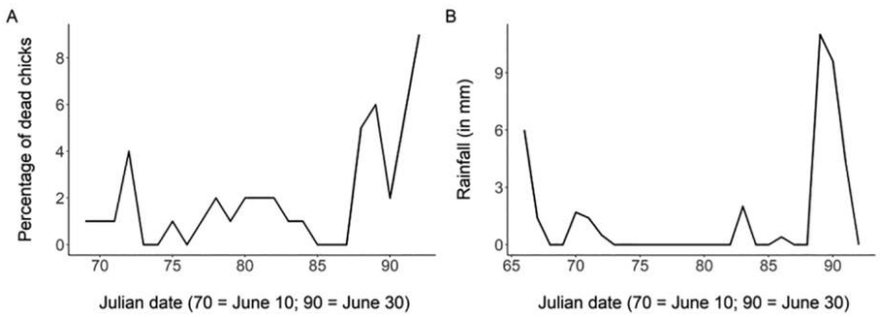

Figure A3. Percentage of dead nestlings in collared flycatchers (*left*) and daily precipitation in the same population (*right*). These graphs show no convincing overlap between the events of continuous rain and the peaks in nestling mortality.

Table A1: Number of days with rain experienced by pied flycatchers in Turku and by collared flycatchers in Gotland during the different periods

Period	Turku	Gotland
	No. days with rain	
Egg laying	6	6
Incubation	12	8
Nestling	9	10
	No. consecutive days with rain	
Egg laying	4 (2 × 2)	2
Incubation	5 (2 + 3)	5 (2 + 3)
Nestling	5 (2 + 3)	6 (2 × 3)

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